APPENDICES

ENGLISH SUMMARY

Fibrosis is a manifestation of chronic and persistent injury and is characterized by imbalanced regulation of extracellular matrix metabolism. This results in abundant production and deposition of extracellular matrix proteins and permanent scarring. Ultimately this leads to irreversible remodeling of injured organs that will eventually impede the physiological function of diseased organs.

Fibrosis in liver or lung tissue is the most prevalent type of fibrosis. Liver fibrosis contributes to more than one million deaths annually via cirrhosis development. The mortality rate of idiopathic pulmonary fibrosis (IPF), the most severe form of lung fibrosis, ranges from 4 to 10 deaths per 100.000 population-years. Until now, few therapeutic options are available for patients with fibrosis and organ transplantation is the only treatment for patients with end-stage fibrosis. Because of that, novel therapeutic approaches to reverse or halt fibrosis and tools to detect or predict the development of disease are urgently needed to provide tailored clinical management for patients.

Fibrosis is characterized by abundant production and deposition of extracellular matrix proteins but several studies have also found that extracellular matrix is not only a consequence but also an important driver of fibrosis development and progression. Based on this, extracellular matrix and associated proteins may therefore be candidates for use as biomarkers or as therapeutic targets in fibrosis. One of these candidates is osteoprotegerin (OPG), which has many matrix-related functions. Recently, serum OPG levels were added to a panel of serum biomarkers called Coopscore® that has promising properties in diagnosing liver fibrosis. Moreover, through interacting with its ligands, receptor activator of nuclear activator kappa-B ligand (RANKL) and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), OPG could have possible profibrotic activities in the development and progression of fibrosis. However little is known about how OPG plays a role in this fibrosis machinery and whether OPG also has the same value as a biomarker in other types of fibrotic disease such as lung fibrosis. Therefore, we aimed to increase our knowledge of the role of OPG, its ligand RANKL and their interactions with extracellular matrix proteins in fibrotic disease development, particularly in liver and lung fibrosis.

In **chapter 3** we found that fibrotic liver tissue produced higher levels of OPG as compared to control liver tissue in both human and murine. Moreover, OPG was expressed locally at the site of fibrotic scars. In addition, TGFβ1, the master regulator of fibrosis, was responsible for the higher production of OPG during fibrosis development. Furthermore, we investigated the role of OPG in this machinery and found that incubation of liver slices with OPG promoted higher expression of fibrotic markers such us procollagen 1α1, αSMA, fibronectin 2 and TGFβ1 mRNA. Interestingly, the profibrotic effect of OPG could be abrogated by galunisertib, a TGFβ receptor kinase inhibitor, implying that TGFβ plays an important role in this event. We also describe that OPG levels were increased in CCL_4-induced liver fibrosis in mice and the higher levels of OPG waned in time after termination

of CCL_4 administration followed by a decrease in scar tissue, implying resolution of fibrosis. Therefore, OPG could possibly be used as a marker to evaluate the efficacy of potential novel drug or treatment candidates against liver fibrosis.

In **chapter 4**, we investigated whether OPG has the same role in other fibrotic disease, particularly in lung fibrosis. Elevated levels of OPG were observed in both human and murine fibrotic lung tissue and abundantly present at the site of active fibrotic development as previously observed in liver fibrosis. Higher production of OPG during lung fibrosis development was also regulated by TGFβ1. Interestingly, although serum OPG levels did not directly discriminate patients with IPF from healthy individuals, higher OPG levels at first hospital presentation were associated with rapid progression of disease. In our small cohort, IPF patients with serum OPG levels higher than 1234 pg/mL progressed faster than patients with lower levels. This is an important early finding since information on the likelihood of IPF progression is essential as it can guide clinicians to tailor clinical treatment of these patients. However, validation with a bigger cohort is warranted to confirm the potential of OPG for determining the prognosis of fibrotic lung disease.

In **chapter 5**, we describe the potential role of OPG and its ligand RANKL in lung fibrosis development. We found that RANKL counteracts lung tissue destruction via promoting alveolar type II (ATII) epithelial cells proliferation and the presence of OPG completely abrogated the ability of RANKL to induce cell proliferation. ATII cells are progenitors for new ATI and ATII cells and known to be an important factor in the lung regeneration and repair after damage. This promising novel role of RANKL may therefore be further explored as a therapeutic approach aiming at stimulating lung tissue repair in lung diseases characterized by inadequate epithelial regeneration like pulmonary silicosis, COPD and cystic fibrosis.

In **chapter 6** we assessed the possible association between OPG with four other important fibrosis-related growth factors or extracellular matrix proteins, i.e. connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), fibulin-1, and collagen 1α1. We found that OPG expression correlates with fibulin-1 in serum and lung tissue of patients with IPF. Interestingly, OPG colocalizes with fibulin-1 in lung fibroblasts and lung tissue of patients with IPF. Moreover, we also observed that knock down of fibulin-1c limits OPG deposition in lung tissue in bleomycin-induced lung fibrosis in mice, but not in lung fibroblasts. This implies that the effect in lung tissue is not via regulation within fibroblasts, but could be in the extracellular region where the two proteins interact. This novel interaction of OPG with fibulin-1 should be further explored to gain insight on how their interaction contributes to lung fibrosis pathogenesis.

To gain deeper insight into interactions between OPG/RANKL and extracellular matrix proteins, in **chapter 7** we explored the impact of pathological stiffness on OPG/RANKL regulation by primary lung fibroblasts. We found that increased stiffness resulted in an increase in OPG and RANKL production along with other fibrosis-related genes such us ACTA2, COL1A1 and fibulin-1 indicating that fibroblasts are triggered and show profibrotic

activity in response to a stiff environment. These findings support our hypothesis about the involvement of OPG in fibrosis development and progression.

To dig deeper into the effects of ECM composition on fibrotic responses or cellular senescence, especially in relation with OPG/RANKL axis, in **chapter 8** we explored whether ECM harvested from senescent and fibrotic fibroblasts can induce fibroblast senescence. We discovered that ECM isolated from fibrotic and senescent fibroblasts did not modulate expression or production of secreted proteins associated with senescence and fibrotic responses in fibroblasts including OPG and RANKL. However, we observer upregulation of DCN and α-SMA gene expression and increase of IL-6, CXCL8, TGF-β1 secretion. Therefore, these data suggest that ECM composition has less impact on cell behavior as compared to ECM stiffness.

Taken together, we conclude that OPG is upregulated as a response to fibrotic stimuli (TGF-β) or changes in ECM stiffness due to fibrosis development, that aberrant production of OPG can be detected in serum of patients with IPF, and that serum OPG levels are linked to progression of lung fibrosis.

NEDERLANDSE SAMENVATTING

Fibrose ontstaat als gevolg van langdurig aanhoudend letsel en wordt gekenmerkt door een disbalans in de regulatie van het extracellulaire matrix (ECM) metabolisme. Dit resulteert in een overmatige productie en afzetting van extracellulaire matrixeiwitten en daardoor blijvende littekens. Uiteindelijk leidt dit tot onomkeerbare beschadiging van organen, waardoor de fysiologische functie wordt belemmerd.

Fibrose in lever- of longweefsel is de meest voorkomende vorm van fibrose. Leverfibrose draagt jaarlijks bij aan meer dan een miljoen sterfgevallen door de ontwikkeling van cirrose. Het sterftecijfer van idiopathische longfibrose (IPF), de meest ernstige vorm van longfibrose, varieert van 4 tot 10 sterfgevallen per 100.000 bevolkingsjaren. Tot nu toe zijn er weinig therapeutische opties beschikbaar voor patiënten met fibrose en is orgaantransplantatie de enige behandeling voor patiënten met fibrose in het eindstadium. Daarom zijn er dringend nieuwe therapeutische behandelingen nodig om fibrose om te keren of te stoppen, en hulpmiddelen om de ontwikkeling van de ziekte te detecteren of te voorspellen om patiënten een op maat gemaakte klinische behandeling te kunnen bieden.

Fibrose wordt gekenmerkt door overvloedige productie en afzetting van extracellulaire matrixeiwitten, maar verschillende onderzoeken hebben ook aangetoond dat extracellulaire matrix niet alleen een gevolg is, maar ook een belangrijke aanjager van de ontwikkeling en progressie van fibrose. Op basis hiervan kunnen extracellulaire matrix en geassocieerde eiwitten daarom kandidaten zijn voor gebruik als biomarkers of als therapeutisch doelwit bij fibrose. Een van deze kandidaten is osteoprotegerine (OPG), dat veel matrixgerelateerde functies heeft. Onlangs zijn serum OPG spiegels toegevoegd aan een panel van serumbiomarkers genaamd Coopscore®, die veelbelovende eigenschappen heeft bij het diagnosticeren van leverfibrose. Bovendien zou OPG door interactie met zijn liganden RANKL en TRAIL mogelijke profibrotische activiteiten kunnen hebben bij de ontwikkeling en progressie van fibrose. Er is echter weinig bekend over hoe OPG een rol speelt in fibrose en of OPG ook dezelfde waarde heeft als biomarker bij andere vormen van fibrotische ziekten zoals longfibrose. Daarom wilden we onze kennis rondom de rol van OPG, zijn ligand RANKL en hun interacties met extracellulaire matrixeiwitten bij de ontwikkeling van fibrotische ziekten vergroten, met in het bijzonder bij lever- en longfibrose.

In **hoofdstuk 3** hebben we gevonden dat fibrotisch leverweefsel hogere niveaus van OPG produceerde in vergelijking met controle leverweefsel in zowel de mens als de muis. Bovendien werd OPG lokaal tot expressie gebracht op de plaats van fibrotische littekens. Daarbij was TGFβ1, de hoofdregulator van fibrose, verantwoordelijk voor de hogere productie van OPG tijdens de ontwikkeling van fibrose. Verder onderzochten we de rol van OPG in het fibroseproces en ontdekten we dat incubatie van leverplakjes met OPG, hogere gen expressie van fibrotische markers stimuleerde, zoals procollageen 1α1, αSMA, fibronectine 2 en TGFβ1. Interessant is dat het profibrotische effect van OPG kan worden opgeheven door galunisertib, een TGFβ-receptorkinaseremmer, wat

impliceert dat TGFβ hierbij een belangrijke rol speelt. We beschrijven bovendien dat OPG niveaus waren verhoogd in CCl_4-geïnduceerde leverfibrose bij muizen en dat de OPG niveaus in de tijd afnamen na beëindiging van CCl_4 toediening, gevolgd door een afname van littekenweefsel, wat impliceert dat fibrose verdwijnt. Daarom zou OPG mogelijk kunnen worden gebruikt als een marker om de werkzaamheid van potentiële nieuwe geneesmiddelen tegen leverfibrose te evalueren.

In **hoofdstuk 4** hebben we onderzocht of OPG dezelfde rol speelt bij andere fibrotische ziekten, met name bij longfibrose. Verhoogde niveaus van OPG werden inderdaad waargenomen in fibrotisch longweefsel (mens en muis) en waren overvloedig aanwezig op de plaats van actieve fibrose ontwikkeling, zoals eerder waargenomen bij leverfibrose. Een hogere productie van OPG tijdens de ontwikkeling van longfibrose werd ook hier gereguleerd door TGFβ1. Interessant is dat hogere OPG spiegels bij geassocieerd waren met snellere progressie van de ziekte, ook al waren serum OPG spiegels van patiënten met IPF op het eerste gezicht niet anders dan die van gezonde individuen. In ons kleine cohort ontwikkelde de ziekte zich sneller in IPF patiënten met serum OPG spiegels hoger dan 1234 pg/ml dan in patiënten met lagere spiegels. Dit is een belangrijke bevinding, aangezien informatie over de waarschijnlijkheid van de progressie van IPF essentieel is, omdat het clinici kan helpen om de behandeling van deze patiënten op maat te maken. Validatie met een groter cohort is echter aanbevolen om dit potentieel van OPG voor het bepalen van de prognose van longfibrose te bevestigen.

In **hoofdstuk 5** beschrijven we de mogelijke rol van OPG en zijn ligand RANKL in de ontwikkeling van longfibrose. We ontdekten dat RANKL de beschadiging van longweefsel tegengaat door de proliferatie van alveolaire type II (ATII) epitheelcellen te bevorderen en dat de aanwezigheid van OPG het vermogen van RANKL om celproliferatie te induceren volledig teniet deed. ATII cellen zijn voorlopers van nieuwe ATI- en ATII cellen en staan bekend als een belangrijke factor bij het herstel van de longen na schade. Deze veelbelovende nieuwe rol van RANKL kan daarom verder worden onderzocht als therapeutische aanpak die gericht is op het stimuleren van longweefselherstel bij longziekten die worden gekenmerkt door onvoldoende epitheliale regeneratie zoals longfibrose, COPD en cystische fibrose.

In **hoofdstuk 6** hebben we het mogelijke verband tussen OPG en vier andere belangrijke fibrose-gerelateerde groeifactoren en extracellulaire matrixeiwitten onderzocht (CTGF, VEGF, fibuline-1 en collageen 1α1). We vonden dat OPG expressie correleert met fibuline-1 in serum en longweefsel van patiënten met IPF. Interessant is dat OPG ook colokaliseert met fibuline-1 in longfibroblasten en longweefsel van patiënten met IPF. Bovendien hebben we gezien dat knock-down van fibuline-1c de afzetting van OPG in longweefsel beperkt in door bleomycine geïnduceerde longfibrose bij muizen, maar niet in longfibroblasten. Dit suggereert dat het effect in longweefsel niet via regulatie in fibroblasten is, maar in het extracellulaire gebied waar de twee eiwitten elkaar beïnvloeden. Deze nieuwe interactie van OPG met fibuline-1 zal verder moeten worden onderzocht om inzicht te krijgen in hoe hun interactie bijdraagt aan het ontstaan en het verloop van longfibrose.

Om beter te begrijpen wat de interacties zijn tussen OPG/RANKL en extracellulaire matrixeiwitten, hebben we in **hoofdstuk 7** de impact van matrix stijfheid op de OPG/RANKL regulatie door primaire longfibroblasten onderzocht. We ontdekten dat verhoogde matrix stijfheid resulteerde in een toename van OPG- en RANKL productie, samen met andere fibrose-gerelateerde genen zoals ACTA2, COL1A1 en fibuline-1, wat aangeeft dat fibroblasten worden geactiveerd en profibrotische activiteit vertonen als reactie op een omgeving met hoge matrix stijfheid. Deze bevindingen ondersteunen onze hypothese over de betrokkenheid van OPG bij de ontwikkeling en progressie van fibrose.

Om dieper in te gaan op de effecten van de ECM samenstelling op fibrotische reacties of cellulaire veroudering, met name in relatie tot de OPG/RANKL as, hebben we in **hoofdstuk 8** onderzocht of ECM gemaakt door verouderde en fibrotische fibroblasten, fibroblastveroudering kan opwekken. We ontdekten dat ECM gemaakt door deze fibroblasten de expressie of productie van uitgescheiden eiwitten geassocieerd met ouderdoms - en fibrotische processen in fibroblasten, waaronder OPG en RANKL, niet beïnvloedde. We observeerden echter wel verhoging van DCN- en α-SMA-genexpressie en toename van IL-6, CXCL8 en TGFβ1-secretie. Daarom suggereren deze gegevens dat de ECM samenstelling minder invloed heeft op de cel dan de stijfheid van de ECM.

Alles bij elkaar genomen concluderen we dat OPG wordt opgereguleerd als een reactie op fibrotische stimuli (TGFβ) of veranderingen in ECM stijfheid als gevolg van de ontwikkeling van fibrose, dat afwijkende productie van OPG kan worden gemeten in serum van patiënten met IPF, en dat deze spiegels zijn gekoppeld aan de progressie van longfibrose.

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Habibie was born in Kendari, Southeast Sulawesi, Indonesia on September 20th, 1983. He obtained his bachelor and pharmacist degree from Universitas Hasanuddin in 2006 and 2007 respectively. After receiving his pharmacist degree, he worked as a lecturer in Faculty of Pharmacy, Universitas Hasanuddin. In 2011, he received DIKTI Scholarship (from Ministry of Research, Technology and Higher Education of the Republic of Indonesia) to pursued a master degree in pharmaceutical sciences at Division of Pathogenic Biochemistry, Institute of Natural Medicine, University of Toyama, Japan. Under supervision of Prof. Ikuo Saiki, Prof. Yoshihiro Hayakawa and Associate Prof. Satoru Yokoyama, he investigated the molecular mechanism of resveratrol induces apoptosis in melanoma cells. In the middle of 2017, he was awarded LPDP scholarship (from Ministry of Finance of the Republic of Indonesia) to continue his PhD study in the Molecular Pharmacology Department, University of Groningen. Supervised by Prof. Barbro Melgert and Prof. Janette Burgess, he studied the osteoprotegerin, RANKL and extracellular matrix intersection in fibrosis. After finishing his PhD study, he will return back to Indonesia and continue to work as a lecturer and researcher in the Pharmacology and Toxicology Laboratory, Faculty of Pharmacy, Universitas Hasanuddin, South Sulawesi, Indonesia.

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LIST OF PUBLICATIONS DURING PHD STUDY

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